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An *Agrobacterium* mediated transformation system of guava (*Psidium guajava* L.) with endochitinase gene

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Abstract – Genetic transformation of guava (*Psidium guajava* L.) was developed for the first time using *in vitro* grown shoot tip explant co-cultivated with *Agrobacterium tumefaciens* strain LBA4404 harbouring binary vector pIIHR-JBMch with endochitinase and nptII genes. The highest transformation efficiency was achieved by wounding explants with tungsten particles (0.5 μ m) through particle acceleration system, followed by infection for 45 minutes with *A. tumefaciens*, grown overnight with 100 μ M acetosyringone, corresponding to $OD_{600}=0.5$ followed by co-cultivation for 72 hours under dark condition on co-cultivation medium (MS+100 μ M acetosyringone+100 mg L^{-1} L-Cystein). Putative transformed explants regenerated shoots on selection medium stressed with 200 mg L^{-1} kanamycin for 12 weeks. Molecular analysis of putative transformants by PCR confirmed the integration of endochitinase and nptII gene in the plant nuclear genome.

Key words: Genetic transformation, *Psidium guajava*, endochitinase, wilt disease.

INTRODUCTION

Guava (*Psidium guajava* L.), an important fruit crop belonging to family Myrtaceae, is cultivated in many tropical and subtropical countries of the world. The world major producers are India, Brazil and Mexico. The economically useful part of guava is the fruit, which is usually consumed raw. Many products are based on the juice and pulp of the fruit. Due to their astringent properties, mature fruits, leaves, roots, bark and immature fruits are used in local medicines to treat gastroenteritis, diarrhoea and dysentery. Production of guava has been affected worldwide by the wilt disease. This soil borne disease was first reported by Das Gupta and Rai (1947). Symptoms of the disease are manifested by the appearance of yellow coloration with slight curling of leaves of the terminal branches. At the later stage, plants show unthriftiness with yellow to reddish discoloration of leaves and subsequently premature shedding. Fruits of all the affected branches remain undeveloped, hard and stony. Later on, the entire plant defoliates and dies. Guava wilt disease is reported to be engendered by *Fusarium oxysporium* f. sp. *psidii*, *F. solani*, *Macrophomina phaseoli*, *Rizhoctonia bataticola*, *Glyocladium roseum* and *Penicillium* fungi.

Substantial research work has been done to control the

fungal wilt disease of guava but so far no conclusive solution has been developed (Misra 2007). In contrast, PCR (Polymerase Chain Reaction) based methods have been used for identification and characterization for *F. oxysporium* f. sp. *psidii* isolates (Mishra et al. 2013a). A wilt resistant rootstock has been developed using *in vitro* cellular selection system in South Africa (Vos et al. 1998). A large number of guava scion varieties have been developed through selection and hybridization. However, a wilt resistant scion guava variety is still lacking.

Currently, there is no report describing the genetic transformation of guava. Alternate approach to control the wilt disease is to develop a transgenic guava plant expressing the endochitinase gene isolated from *Trichoderma harzianum* (Saiprasad et al. 2009). The technique of expressing the endochitinase gene in the plant system to confer resistance against fungal diseases has been successfully demonstrated in apple (Bolar et al. 2000), cotton (Emami et al. 2003, Cheng et al. 2005), broccoli (Mora and Earle 2001), lemon (Gentile et al. 2007) and rice (Lu et al. 2004, Shah et al. 2009). Insertion of genes encoding hydrolytic enzymes, which can degrade fungal cell wall such as chitinase and glucanase, could pave the way for developing wilt resistant

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guava variety. In this study we describe the development of an efficient transformation system from *in vitro* grown shoot tips of guava.

MATERIAL AND METHODS

Plant material

In vitro shoot tips were induced from mature shoot explant of guava cv. Lalit, an improved guava selection having red pulp, through enhanced axillary branching under *in vitro* condition. Shoot tips were proliferated on MS (Murashige and Skoog) medium fortified with 3 mg L⁻¹ BAP (6-Benzylaminopurine), as per the protocol described by Mishra et al. (2007).

Agrobacterium tumefaciens strain and plasmid

Genetic transformation was performed using the *A. tumefaciens* strain LBA4404 (Ooms et al. 1981) harbouring the binary vector pIIHR-JBmch with full length endochitinase gene obtained from *Trichoderma harzianum*, neomycin phosphotransferase (nptII) gene under the control of CaMV 35S promoter and Nos terminator (Saiprasad et al. 2009).

Wounding of the explant

The shoot tip explants (0.5 cm) were wounded with three different methods, in order to enhance transformation efficiency. In the first experiment, around 50 mg autoclaved silicon carbide (600 mesh) was poured in polypropylene tubes along with 5 ml sterilized MS salt solution. Then the shoot tips were suspended in aliquot. The suspension was vortexed for 5 minutes at room temperature. The injured shoots were placed in MS salt solution until agro infection. In the second experiment, the explants were subjected to particle bombardment using uncoated, sterile tungsten particles of 0.5 µm size (Sigma, USA), suspended in a solution of MgSO₄ (10 mM) and spermidine (10 mM). Particle bombardment was carried out by using ballistic gene gun or particle acceleration system (Gene Pro HE-2000, India) with He pressure of 12 kg cm⁻². In the third experiment, shoots were manually wounded (notching) using sterile dissection needle.

Infection and co-cultivation

Fresh culture of *A. tumefaciens* was prepared by overnight growth at 28 °C in YEB medium containing 50 mg L⁻¹ kanamycin and 25 mg L⁻¹ rifampicin. The bacterial culture was centrifuged at 5000 rpm for 10 minutes and then the pellet was resuspended in MS liquid medium to obtain a density of 0.5 (OD₆₀₀=0.5). Micro wounded shoot tips were infected with *Agrobacterium* suspension containing 100 µM

acetosyringone for 30, 45 and 60 minutes under mild agitation (100 rpm). After blotting away the excess of bacterial culture, the explants were placed again on co-cultivation medium (MS+100 µM acetosyringone+100 mg L⁻¹ L-Cystein) for 24, 48 and 72 hours under dark conditions at 25±2 °C.

Selection and regeneration of putative transformants

After co-cultivation, the explants were washed thrice with MS salt containing cefotaxime 500 mg L⁻¹ and were blotted dry using sterile blotting paper. The *Agrobacterium* infected shoot apices were selected on MS medium containing 2 mg L⁻¹ BAP + 0.1 mg L⁻¹ IAA (Indole-3-acetic acid) and different concentration of kanamycin (50, 100, 200, 300 mg L⁻¹) for 12 weeks. The survival of explant under different kanamycin regimes were recorded and statistically analyzed.

Rooting and acclimatization

The selected shoots (2 cm) were rooted on MS medium fortified with 1 mg L⁻¹ IBA (Indole butyric acid) + 500 mg L⁻¹ activated charcoal + 200 mg L⁻¹ kanamycin. The rooted plantlets were successfully acclimatized on sterilized coco peat fortified with MS solution under *in vitro* condition. The plants were subsequently shifted to containment facility.

PCR analysis

Total genomic DNA was extracted from the leaves of the individual putative transformed plants using Qiagen's Plant DNA extraction kit (Qiagen, USA). Quality of the genomic DNA was analyzed by electrophoresis on 0.7% agarose gel. PCR analysis of putative transgenic plantlets was performed to confirm the integration of the transgene into the plant genome. Individual PCR analysis was performed using 100 ng DNA, 1 U *Taq* DNA Polymerase, 0.5 µM of each primers, 0.2 mM dNTPs, and 1x PCR buffer in a final volume of 20 µl. NPTII (Forward: 5'-TCTCACCTTGCTCC TGCC-3' and Reverse: 5'-AGGC-GATAGAAGGCGATG-3') and endochitinase gene specific primers (Forward: 5'-TTAATTTGTTACGGAATCATAGA-3' and Reverse: 5'-T TGAGACCGTTTCGGATGTT-3') were used for PCR. PCR was performed on Bio-Rad thermal cycler (USA) for 35 cycles at 94 °C for 1 minute, 54.4 °C for 1 minute and 72 °C for 1 minute for nptII gene specific primers. For endochitinase gene specific primers, 30 cycles at 94 °C for 1 minute, 53 °C for 1 minute and 72 °C for 2 minutes were performed. Both the reactions were preceded by a primary denaturation step at 94 °C for 10 minutes and followed by final extension at 72 °C for 5 minutes.

Genomic DNA extracted from non-transgenic plant was taken as negative control and plasmid DNA of pIIHR-JBMch harbouring the endochitinase gene was taken as positive control. Samples were analyzed by electrophoresis on 1% (w/v) agarose gel containing ethidium bromide and visualized on UV transilluminator.

Statistical analysis

The experiment was conducted in a completely randomized design with three replications and each replicate was inoculated with 50 shoot explants. The angular transformation was done on the data to get the valid conclusions. The various components of the variations were partitioned using ANOVA at 5% significance level and further the data was subjected to Duncan's Multiple Range Test to arrive at pair wise comparisons between the treatment means.

RESULTS AND DISCUSSION

Guava (*Psidium guajava* L.) is an important commercial fruit crop. However, wilt disease caused by *Fusarium oxysporum*, a soil borne fungus is a serious threat to its production (Mishra et al. 2013b).

Effect of wounding methods on transformation efficiency

In vitro grown shoot tips (0.5 cm) of guava cultivar Lalit were subjected to wounding. Out of three different methods tried to induce wound, shoot tip explants bombarded with sterile tungsten particles (0.5 μ m, Sigma, USA) using

acceleration system (Gene Pro HE-2000, India) with He pressure of 12 kg cm⁻² was found best in terms of enhancing number of putative transformants (4.66%). The transformation efficiency was reduced when explant were wounded with silicon carbide (2%). Lowest transformation efficiency (1.33%) was observed with explants wounded by notching of meristem. However, no transformation was observed in non wounded explants (Table 1). Wounding is known to trigger the secretion of some phenolic compounds from wound site of dicotyledonous plants, which subsequently increased the transformation efficiency (Potrykus 1990). Recently, Pradhan et al. (2013) also reported that wounding was prerequisite in *Agrobacterium* mediated transformation in *Dalbergia sissoo*.

Agrobacterium is attracted to a wounded plant in response to phenolic compounds such as acetosyringone and α -hydroxyl-acetosyringone released by plant cells to which they become attached (Zambryski 1992). These phenolic compounds activate vir regulon present in Ti plasmid of *A. tumefaciens* (Stachel et al. 1985). Microprojectile bombardment is known mainly as a tool for direct gene transfer (Bidney et al. 1992, Klein and Fitzpatrick 1993). It is clear from our results that wounding induced by tungsten mediated microprojectile bombardment yielded high transformation efficiency in guava compared to silicon carbide mediated explant wounding. In fact, after microprojectile mediated wounding, 5.33 per cent of the inoculated stem explants were transformed. The observed efficiency and reproducibility may be due to the microprojectile's high velocity and

Table 1. Effect of wounding methods on transformation efficiency

Wounding methods	No. of explant targeted	Kanamycin resistant shoots (%)	Transformation efficiency (%)
No wounding	150	0.00±0.00 ^a	0.00±0.00 ^a
Silicon carbide mediated wounding	150	3.00±0.57 ^b	2.00±1.15 ^{ab}
Tungsten mediated microprojectile wounding	150	6.33±0.88 ^c	4.66±0.6 ^b
Notching of the shoot meristem	150	2.00±0.57 ^b	1.33±0.6 ^{ab}

Mean values in each column with different letters (in superscripts) are significantly different at ($p < 0.05$; Duncan's New Multiple Range Test).

Table 2. Effect of infection and co-cultivation duration on transformation efficiency of guava

Infection time (minutes)	Co-cultivation time (Hours)	No. of explant targeted	Kanamycin resistant shoots (%)	Transformation efficiency (%)
30	24	150	0.00±0.00	0.00±0.00
	48	150	0.33±0.33	0.66±0.66
	72	150	0.66±0.33	1.33±0.66
45	24	150	1.00±0.00	2.00±0.00
	48	150	1.33±0.33	2.66±0.66
	72	150	2.66±0.33	5.33±0.66
60	24	150	0.66±0.33	1.33±0.66
	48	150	0.33±0.33	0.66±0.66
	72	150	0.00±0.00	0.00±0.00

small size, as well as their relatively uniform spreading in the bombarded tissue (Zuker et al. 1999).

Effect of infection and co-cultivation duration

Infection and co-cultivation duration had a marked effect on transformation frequency. Data clearly indicates that maximum transformation frequency (5.33%) was achieved by infecting the shoot tip explants with *Agrobacterium* for 45 minutes. Reducing (30 minutes) or enhancing (60 minutes) infection duration resulted in low transformation efficiency (Table 2). Co-cultivation of agro-infected shoots for 72 hours under dark was found optimum for enhancing transformation efficiency (5.33%). However, reducing (24, 48 hours) co-cultivation period resulted in low transformation efficiency (Table 2). It has been proposed that during infection explant undergoes a physiological and developmental shift to enter for morphogenic competency and when the T-DNA is inserted following this short period the recipient cells enter the regeneration pathway (Agarwal et al. 2004). Our results clearly indicated that 45 minutes of infection of overnight grown *Agrobacterium* and 72 hours co-cultivation in the presence of 100 μ M acetosyringone under dark gave highest transformation efficiency. Archiletti et al. (1995) reported infection time of 30 minutes in almond. Ogaki et al. (2008) also reported an optimum co-cultivation period of three days for genetic transformation of *Lilium*. Co-cultivation period of four days has been reported for *Galega orientalis* (Collen and Jarl 1999), five days for *Dalberia sissoo* (Pradhan et al. 2013), whereas co-cultivation for two days was best for several other legumes (Husnain et al. 1997). These variations in the requirement for a definite/specified co-cultivation period could perhaps owe to plant tissue specificity.

Selection of putative transformants

Highest number of putative transformants (5.33%) was obtained on MS medium supplemented with 200 mg L⁻¹ kanamycin + 2mg L⁻¹ BAP + 0.1 mg L⁻¹ IAA (Figure 1). At 300 mg L⁻¹ kanamycin, the phytotoxic effect was noticeable as explants started to bleach in 30 days after inoculation at 12 weeks (Table 3). However, at lower selection pressure (50

mg L⁻¹) kanamycin escape was observed and 80% plants survived even after 12 weeks of inoculation. The transformed shoots were rooted on MS medium fortified with 1 mg L⁻¹ IBA + 500 mg L⁻¹ activated charcoal. The rooted putative transformants were acclimatized on autoclaved coco peat supplemented with MS salt solution. It has been observed that higher kanamycin concentration is required for selection of transformants in guava. This may be attributed to the explant type and tissue specificity.

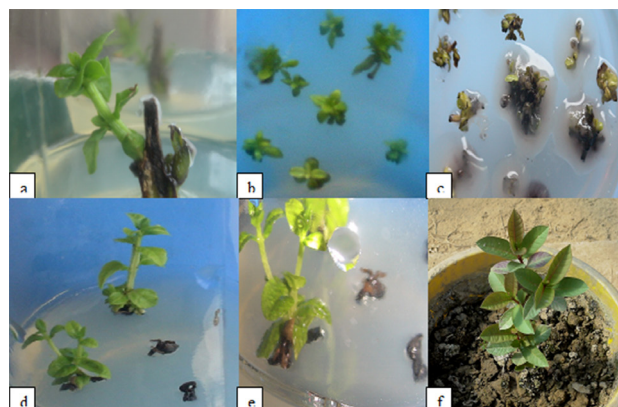


Figure 1. *In vitro* selection of putative transformants of guava: a) *In vitro* bud induction in guava, b) pre-culture of explant, c) co-cultivation of explant with *Agrobacterium*, d) selection of explant in kanamycin 200 mg L⁻¹ in 8 weeks, e) selection of explant in kanamycin 200 mg L⁻¹ in 12 weeks, f) acclimatized guava plant.

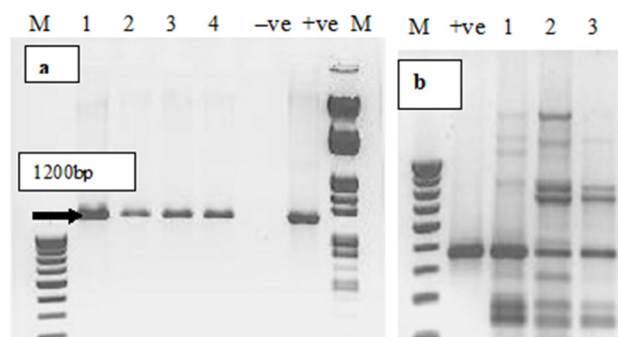


Figure 2. PCR confirmation of transformed plants: a) Lines 1-4 showing transformed plant analyzed for presence of endochitinase gene (1200 bp), (b) Lines 1-4 showing transformed plant analyzed for npt II gene +ve: positive control, -ve: negative control, M: 100bp DNA ladder (Marker).

Table 3. Effect of different doses of kanamycin on selection of putative transformants

Kanamycin (mg L ⁻¹)	No. of explant targeted	No. of explant survived after 4 weeks	Survival of explant after 8 weeks	Survival of explant after 12 weeks
50	50	48.00±0.57 ^a	46.33±0.88 ^a	42.66±0.66 ^a
100	50	43.66±1.20 ^a	39.33±1.33 ^b	35.00±1.52 ^b
200	50	28.00±2.30 ^b	13.33±2.40 ^c	5.33±0.66 ^c
300	50	35.33±1.76 ^c	7.00±0.57 ^d	0.33±0.33 ^d

Mean values in each column with different letters (in superscripts) are significantly different at ($p < 0.05$; Duncan's New Multiple Range Test).

PCR analysis

On the basis of kanamycin tolerance, a total number of 16 plants were selected and subjected to PCR analysis. The genomic DNA was isolated from the leaf tissues of the kanamycin selected plants and PCR was performed to confirm the integration of gene. A reaction containing pIIHR-JBMch template served as positive control and leaf genomic DNA isolated from a non transformed naturally occurring plant of *P. guajava* L. served as the negative control. Agarose gel profile representing the PCR products showed amplicon of ~480 bp size of nptII gene. Similarly, endochitinase gene specific primers resulted in amplification of ~1200 bp fragment (Figure 2). The results of PCR confirmed the successful transfer and integration of endochitinase and nptII genes in 14 plants from the T-region of the vector into the plant nuclear genome.

Establishment of transgenic plants in containment facility

Well rooted 13 week old transformed plantlets were removed from the culture medium and the roots washed gently under running tap water to remove the adhering agar and transferred to autoclaved coco peat supplemented

Sistema de transformação de goiaba (*Psidium guajava* L.) com gene endoquitinase mediado por *Agrobacterium*

Resumo – Transformação genética de goiaba (*Psidium guajava* L.) foi desenvolvida pela primeira vez usando explante de broto crescido in vitro, co-cultivado com linhagem LBA4404 de *Agrobacterium tumefaciens* abrigando o vetor binário pIIHR-JBMch com os genes de endoquitinase e nptII. A mais alta eficiência de transformação foi obtida por bombardeamento de explantes com partículas de tungstênio (0.5 µm) através do sistema de aceleração de partículas, seguido por infecção por 45 minutos com *A. tumefaciens*, crescido durante a noite com 100 µM de acetosiringona, correspondendo a $OD_{600}=0.5$ seguido por co-cultivo por 72 horas sob escuro em meio (MS+100 µM acetosiringona+100 mg L⁻¹ L-Cisteína). Explantes transformados regeneraram brotos em meio seletivo com 200 mg L⁻¹ de kanamicina por 12 semanas. Análise molecular dos transformantes por PCR confirmaram a integração dos genes de endoquitinase e nptII no genoma nuclear da planta.

Palavras-chave: transformação genética, *Psidium guajava*, endoquitinase, murca da goiabeira.

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with MS salt solution, moistened with autoclaved water. The potted plants, covered with Polyethylene bags were maintained under biosafe containment facility at 25±1 °C and 85-95% RH for four weeks, which were subsequently shifted to polyhouse.

Our results present an efficient methodology for high frequency genetic transformation of *P. guajava* plants demonstrating transgene integration and their expression in terms of kanamycin tolerance. The highlight of the study include microprojectile mediated wounding of explant and stringent selection strategy which allowed high frequency production of transformed plants coupled with a low rate of “escape” (false positive).

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